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Yersinia pestis Metabolic Network

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Yersinia: Systems biology and Control

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Metabolism of *Yersinia pestis*

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Abstract

Bubonic plague is one of the deadliest diseases known to man. Unfortunately, despite all of our medical advances, we still do not have a working vaccine against this disease. Worse yet, discovery of anti-microbial resistant strains of *Yersinia pestis*, the causative agent of plague, could soon render our current therapeutic means ineffective. Unique characteristics of bacterial metabolism constitute one of the primary sets of targets for drug design. Accordingly, metabolism of *Y. pestis* has been one of the most studied aspects of its physiology.

In this chapter we aim to provide a summary report of the current knowledge about this bacterium's metabolism. We report on the *Y. pestis*' evolutionary efforts to downsize its metabolism as it adapts to environments and modes of transmission that are different from that of its progenitor. We detail the known changes in *Y. pestis*' metabolism as it transitions between different stations of its lifecycle. We describe the role of metabolism in bacterial virulence. Finally, we report the results of system-level analyses of *Y. pestis* that use the available genomic and transcriptomics data to assess the organism's response to various genetic and environmental perturbations.

I. Introduction

Bacteria, as with all life forms, need to import and transform nutrients from their surroundings. These compounds are consumed to provide the cell with building blocks

and energy needed for maintenance of necessary operations and production of biomass. Despite many similarities in the basic chemistry of metabolism, the enzymatic means to achieve these transformations can vary among different organisms. One of the primary methods of drug target selection involves exploiting these differences for therapeutic purposes when they manifest between metabolic pathways of a host and malignant cells. By specifically targeting the unique metabolic pathways of invading bacteria (e.g. (Gerdes et al., 2002; Bhave et al., 2007)) or cancer cells (e.g. (Christofk et al., 2008; Boxer et al., 2010; Tennant et al., 2010)), one can synthesize drugs that will incapacitate these exceptional features while sparing similar functionalities in normal host cells.

In the case of *Yersinia pestis* (YP), the etiological agent of bubonic plague, two primary factors propel the need for system-level metabolic analyses. The first is the possibility of identifying metabolic weak points that are unique to this organism and that can be exploited for therapeutic purposes. The second impetus entails answering the intriguing question of how an offshoot of *Yersinia pseudotuberculosis* (YPS) (a food-borne gastrointestinal bacterium that causes chronic diarrhea, fever and abdominal pain) can mutate to cause bubonic plague, one of the deadliest diseases known to man. The latter objective is one of the main reasons why metabolism of YPS has been studied almost to the same extent as that of YP and why nearly every metabolic analysis of YP's metabolism compares its phenotypic capability to that of YPS. Such analyses can greatly aid in the identification of the evolutionary objectives of YP and possibly even predict future mutations. These phenotypic analyses gain even greater importance once one considers that genomic analyses show that these two organisms are almost

identical (greater than 90% chromosomal similarity and 99.7% nucleotide sequence match in 16S rDNA (Bercovier et al., 1980; Ibrahim et al., 1993). Therefore, very few changes have had very great consequences.

The advent of field of systems biology, based mostly on great success of functional genomics, has revolutionized the way treatment targets are identified. There has been a canonical shift in antimicrobial drug discovery from phenotypic screening against large libraries of compounds to that of pinpoint chemical inhibition of genome-based preselected targets (Freiberg and Brötz-Oesterhelt, 2005; Pucci, 2006). High throughput analyses have identified large numbers of possible essential genes. In some cases nearly 25% of the genetic complement has been labeled as critical for cellular viability (Trawick and Schilling, 2006). However, experimental gene knockout analyses have found that the actual percentage of critical genes usually range between 6 to 17% of cellular genome (Giaever et al., 2002; Kobayashi et al., 2003). Note that the particular nutrient environment may, in general, significantly change these percentages (Papp et al., 2004; Pal et al., 2006). However, it can be concluded that although bioinformatic and high-throughput methods can narrow the list of potential drug targets, the ultimate proof of “essentiality” of a gene can only be verified through its directed knockout. Furthermore, if the research aims include development of broad-spectrum therapeutics, it is necessary that the essentiality of orthologs in a number of similar organisms also be experimentally verified.

The proven panacea for eliminating a large fraction of the predicted false-positive essential genes entails incorporating disparate kinds of information such as data on cellular transcriptomics and proteomics, as well as kinetic capacity of various enzymes,

into system-level studies. Similarly, any detailed review of a bacterium's metabolism should focus on recent genome-based analyses as well as experimental observations that have been made using trusted traditional methods.

In case of YP and other Yersiniae, our understanding of their metabolism originates from four complimentary sources. First, knowledge regarding various metabolic phenotypes stems from numerous detailed studies that were conducted as early as the first half of the twentieth century. These studies primarily examined *in vitro* catalytic capabilities of cell extracts under a variety of different conditions. Additionally, these studies investigated YP's metabolic requirements and preferences for nutrients. Some of these studies quantified the import and export of metabolites under a variety of different conditions using an assortment of YP strains (both virulent and avirulent). A comprehensive review of these early studies can be found in Pollitzer 1960 (Pollitzer, 1960).

The second foundation of our insights into metabolism of YP is the availability of annotated genomes for more than ten[1] different strains of Yersiniae, representing the four YP biovars as well as strains of YPS and Yersinia enterocolitica (YPE). Extensive genomic comparisons (Chain et al., 2004; Chain et al., 2006; Gu et al., 2007) have shed new light on the genetic bases for some of the earlier observations. These studies have also identified gaps in our knowledge which need to be filled. Genomic information has been most informative in drawing attention to specific functional losses by YP which point to the organism's adaptation to a new lifestyle. Furthermore, analyses of genetic

[1] At the time of preparation of this manuscript, the annotated genomes of twelve strains of Yersinia were available on the Kyoto Encyclopedia of Genes and Genomes.

acquisitions that encode virulence factors have improved our understanding of the sequence of events which lead to host infection.

Furthermore, the availability of annotated genomes allows for the development of system-level models of metabolism which permit the researcher to a) computationally assess the robustness of bacteria to various genetic mutations, b) analyze flux patterns in the metabolic reactions, c) evaluate the redundancy of metabolic pathways, d) determine the bacterium's minimal nutritional requirements and metabolic efficiency, and e) help improve genome annotation by identifying silent genes and orphan enzymes. To date, only one genome-scale model of metabolism in YP (strain 91001, biovar microtus) has been published (Navid and Almaas, 2009). However, models for strains representing the other three biovars as well as YPS have been developed and will be published in the near future (Navid and Almaas, submitted).

Finally, with the advent of high throughput microarray technology, a number of studies have been conducted in order to examine the differences in levels of gene-expression in YP as it adapts to different environments and modes of stress. Some of these studies have examined YP's response to: changes in temperature and nutritional availability (Han et al., 2004; Motin et al., 2004), heat and cold shock (Han et al., 2005), and interactions with antibiotics (Qiu et al., 2005; Qiu et al., 2006). These analyses provide the researcher with added insights into augmentations in cellular priorities in response to external threats. These changes usually involve activation of defense mechanisms which can be very taxing on cellular metabolism and in most cases result in depletion of cellular energy charge (Zahorchak et al., 1979; Corton et al., 1994; Finkel and Holbrook, 2000; Tiwari et al., 2002).

Here we have tried to gather from these four sources, the bulk of what is known about metabolism in YP. We begin by detailing proposed causes for metabolic changes as they pertain to evolution of YP and its capacity to infect hosts. We have divided cellular metabolism based on popular designations of metabolic pathways. Finally, we have provided characteristic details of the *in silico* model of metabolism in YP as well as a new computational tool, CryptFind, which can be used to identify genes that are responsible for variations in cellular phenotype depending on environmental conditions.

II. Divergence from progenitor

Sometime after the end of last ice age (1500-20000 years ago), *Y. pestis* began a rapid divergence from *Yersinia pseudotuberculosis* (Achtman et al., 1999). Genomic comparisons of the annotated genomes between YP and YPS have found very few YP specific genes. Most of the genetic differences between the two organisms involve inactivation of YPS genes (approximately 13% of the genome) (Chain et al., 2004). These loses can be attributed to the fact that, in contrast to the chromosome of its progenitor, YP's genome is replete with inversions, transpositions and additions/deletions (Parkhill et al., 2001; Deng et al., 2002; Zhou et al., 2004a; Zhou et al., 2004b; Chain et al., 2006). Analyses of the nucleotide sequence of YP (strain CO92) have found at least 149 cryptic/pseudogenes (~4% of the genome). Pseudogenes are genes that have been inactived, usually due to point mutations that result in a frame shift in the coding sequence. Cryptic genes, as with pseudogenes, are also inactive under normal circumstances. However, if environmental conditions necessitate their functionalities, these genes have the capability to regain their activity through simple mutations. Presence of cryptic genes in YP eliminates the metabolic

burden of producing proteins that the bacterium does not need for its normal life cycle, however, it provides the organism with a reservoir of determinants which can be acquired if the subsequent need should arise. Identification of these genes is critical for development of therapeutic measures. A new computational tool called CryptFind (Navid and Almaas, 2009) has been developed which utilizes constraint-based modeling approaches and normal phenotypic characteristics to identify candidate cryptic genes. The details of this method are discussed in a later section.

Based on whole genome analyses, there are 2568 genes that are similar between different strains of YP but differ from YPS (Gu et al., 2007). A significant fraction (>40%) of these mutations are associated with cellular metabolism and thus indicate an evolutionary strategy to transition from a free-living organism to one that is dependent on a stable and nutrient-rich environment. Furthermore, strain specific analyses of gene-inactivations and deletions have shown that the majority of these strain-specific functional loses include proteins that are involved in the interactions of bacteria with their environment (Chain et al., 2006). These include membrane proteins, flagellar proteins, chemotaxis proteins, and ABC transporters. Thus, it can be reasoned that the differences in functional reductions between different strains of YP reflect the diversity in geographical and environmental niches that these organisms occupy. It is obvious that some of the lost functions are those that are essential for survival of saprophytes in natural environments and are not needed for survival of an organism whose lifecycle in the flea vector and *in vivo* does not place it in such austere settings.

Aside from adaptation to a new environment, some of the metabolic losses are directly linked to bacterial virulence. In some organisms it has been shown that the inactivation

of select genes increases bacterial virulence (Nakata et al., 1993; Maurelli et al., 1998; Parish et al., 2003). Metabolic comparisons between YP and its forbearer indicate that some of the lost functionalities echo the differing routes of infection between the two organisms. For example, some of the genes associated with enteric infection have been inactivated. These include the genes responsible for expression of the urease enzyme (UreD) (Sebbane et al., 2001) which is needed for oral transmission and survival in ascetic natural environment. Similarly, the genes *yadA* and *inv* have been inactivated. The products of these genes enable YPS to hold on to the surface of intestines and infect the lining of epithelial cells (Rosqvist et al., 1988). Table 1 lists some of the metabolic differences between most strains of YP and YPS.

The lost functionalities have been supplemented by acquisition of new genes which are critical for intradermal injection, virulence and host-infection. Two plasmids present in YP and missing in YPS have been labeled pPCP (~10 kb) and pMT (~100 kb) (Hu et al., 1998). The former encodes plasminogen activator which is required for the bacterial dissemination after subcutaneous injection (Sodeinde et al., 1988; Lahteenmaki et al., 1998; Sebbane et al., 2006). The pMT plasmid encodes for murine exotoxin and F1 capsular antigen. These virulence factors play important roles in bacterial transmission from fleas (Hinnebusch et al., 1996; Hinnebusch et al., 1998; Hinnebusch et al., 2002). The former is required for survival of YP in the highly antibacterial environment of flea proventriculus (Hinnebusch et al., 1998), while the latter has been shown to grant significant but not indispensable protection against incorporation by mouse professional phagocytes (Burrows, 1957).

Determinant	Reference
Glucose 6-phosphate dehydrogenase	(Mortlock and Brubaker, 1962)
Aspartase	(Dreyfus and Brubaker, 1978)
Biosynthesis of glycine/L-threonine	(Burrows and Gillett, 1966)
Biosynthesis of L-phenylalanine	(Burrows and Gillett, 1966)
Biosynthesis of L-methionine	(Burrows and Gillett, 1966)
Rhamnose Fermentation	(Englesberg, 1957a)
Melibiose Fermentation	(Pollitzer, 1954)
Urease	(Sebbane et al., 2001)

Table 1. Metabolic losses by typical strains of YP following their divergence from YPS.

III. Biovar designation based on metabolic differences

Dissimilarities in metabolic capabilities have been used to divide strains of YP into four separate biovars (Devignat, 1951; Zhou et al., 2004b). The metabolic characteristic of each biovar are shown in Table 2. The biovar antiqua which originated from Africa and is believed to have caused the great Justinian plague of 6th century CE can ferment both glycerol and arabinose as a carbon source while simultaneously reducing nitrate to be used as a nitrogen source.

The biovar mediaevalis originated from central Asia and has been associated with the infamous Black Death pandemic that devastated the population of Europe in the 14th century CE. Due to a frameshift mutation in the *napA* gene which translates into periplasmic nitrate reductase (EC 1.7.99.4), this biovar lacks the capability to utilize nitrate as a nitrogen source (Deng et al., 2002). Biovar orientalis originates from southern China. This biovar is widespread, and at the present is the only biovar that is found in the western hemisphere. These strains are associated with modern plague outbreaks. Unlike other biovars, these strains lack a functional glycerol-3-phosphate dehydrogenase enzyme (EC 1.1.5.3). This is due to a 93 bp deletion of the *glpD* gene. This loss prevents YP strains of this biovar from utilizing glycerol as a carbon source

Biovar	glycerol fermentation	arabinose utilization	nitrate reduction
antique	✓	✓	✓
mediaevalis	✓	✓	✗
orientalis	✗	✓	✓
microtus	✓	✗	✗

Table 2. Metabolic characteristics of biovars of YP

(Parkhill et al., 2001; Motin et al., 2002). It is worth noting that these metabolic differences have been shown to have no relationship to the degree of virulence of the organism and that the association with each pandemic is derived from examination of historical records and epidemiological data (Devignat, 1951; Guiyoule et al., 1994).

The recently discovered biovar of YP that has been labeled microtus (Zhou et al., 2004b) is an offshoot of the mediaevalis biovar. The strains in this biovar are avirulent to humans and other large mammals but are lethal to a number of small rodents. As with mediaevalis, this biovar of YP cannot reduce nitrate. Additionally, it is incapable of fermenting arabinose as an energy source. This unique metabolic shortcoming has been attributed to a mutation in the *araC* gene which encodes a regulatory protein that in presence of arabinose activates the P_{araBAD} promoter for enzymes needed for arabinose fermentation (Zhou et al., 2004b).

IV. System level analysis of metabolism

Availability of genomic information has led to a paradigm shift in microbiology from exhaustive analyses of local and limited cellular details to system-level analyses of the organism as a whole. The theoretical models associated with the former utilize hard to

obtain and therefore sparsely collected kinetic parameters to mathematically describe interactions among a small clique of reactions. As can be expected, due to a dearth of kinetic and metabolomic data, such detailed modeling is infeasible for genome-scale analyses.

However, new constraint-based computational methodologies have been developed which forgo some level of detail (such as insights into transient behavior of metabolites and enzyme-substrate affinity) in order to gain a broader understanding of overall metabolic capabilities of a cell. The most successful of these constraint-based approaches is called flux balance analysis (FBA) (for reviews see (Price et al., 2004; Feist et al., 2009; Oberhardt et al., 2009)). FBA modeling is based on the knowledge of the stoichiometry of metabolic reactions which can easily be extracted from the annotated genomes. To date, genomes of a number of YP strains, representing the four biovars have been sequenced and annotated. These include strain 91001 (microtus) (Song et al., 2004), Antiqua (antiqua) (Chain et al., 2006), CO92 (orientalis) (Parkhill et al., 2001), KIM (mediaevalis) (Deng et al., 2002), and Nepal516 (antiqua) (Chain et al., 2006).

Additionally, FBA models require a priori knowledge of an organism's nutritional requirements. In the case of YP, there is a wealth of data available for modeling purposes (e.g. (Higuchi and Carlin, 1958; Higuchi et al., 1959; Smith and Higuchi, 1959; Burrows and Gillett, 1966)). These data are used to constrain cellular growth and the uptake of nutrients and export of waste materials. The constraints also limit the cellular energy metabolism to a narrow set of possible catabolic pathways.

Based on this information, FBA models aim to optimize a cellular task (an objective function) while calculating steady state flux patterns for metabolic reactions that adhere to constraints imposed on the system by mass balance, the structure of metabolic networks, as well as nutritional characteristics of the growth medium. The conventional objective function is growth, although other choices are possible depending on the selective environment of the cell (Schuetz et al., 2007).

FBA has successfully been employed to develop genome scale models of a number of organisms (e.g. (Reed et al., 2003; Duarte et al., 2004; Mahadevan et al., 2006)). These models have been used to study a variety of topics ranging from effects of genetic mutations on cellular metabolism (Fong and Palsson, 2004) to finding new regulatory interactions (Herrgard et al., 2006) and system-level order of metabolic fluxes (Almaas et al., 2004; Almaas et al., 2005). In a similar manner, in order to gain a better understanding of metabolism in YP, the available annotated genome of strain 91001 (Song et al., 2004) has been used to develop a system-level model (iAN818m) of metabolism in YP (Navid and Almaas, 2009). The genome includes 4037 genes, of which, less than a third (1146 genes) are associated with cellular metabolism. Model iAN818m accounts for the activity of 818 of these genes (71%) that result in 969 enzymatic reactions. Further inspection of the literature identified the activity of 37 local orphan enzymes (20-based on literature, 4-pathway hole-filling, 13 critical for biomass production) and 14 non-enzymatic reactions. Thus, the final tally for the model includes 1020 reactions and 825 metabolites.

Metabolic Pathway	Associated reactions
Amino acid	200
Central carbon	201
Cofactor	159
Energy	27
Membrane	134
Nucleotide	123
Other	34
Transport	142

Table 3. Grouping of metabolic reactions in model iAN818 (Navid and Almaas, 2009) based on pathways.

As will be discussed later, metabolism of YP is drastically altered upon its transfer from flea to mammalian host (Han et al., 2004; Motin et al., 2004). A number of studies have shown that this transition modifies the composition of YP's cellular membrane (Kawahara et al., 2002; Knirel et al., 2005; Knirel et al., 2006). The FBA model accounts for this transition by utilizing a different biomass composition for each environment. Finally, the model incorporates another unique feature of YP metabolism by including the crucial pathway for biosynthesis of the virulence factor yersiniabactin. The breakdown of the reactions in iAN818m based on metabolic pathway affiliation is shown in Table 3.

1. Meiotrophy

Early analyses of biosynthetic capabilities of YP revealed that some mutant strains of the bacterium displayed added determinants which were absent in the wild type organism. Ellis Englesberg labeled these mutants which required fewer growth factors than the wild type strain as "meiotrophic" (Englesberg and Ingraham, 1957). Table 4 lists some of the known meiotrophic pathways of YP. A comparison of Table 1 and Table 4 shows that large of number of metabolic determinants that have been lost by YP since its divergence from YPS behave meiotrophically. It is unknown whether

Metabolic determinant	Known or candidate cryptic genes
Biosynthesis of gly/thr	*glyA*[2]*,metL, thrB, thrC*
Biosynthesis of val/ile	*ilvC, ilvD*
Biosynthesis of phe	**pheA**
Biosynthesis of met	**metB**
Urease	**ureD**
Fermentation of arabinose	**araC**
Fermentation of melibiose	**melB**
Fermentation of rhamnose	*betB, rhaA, rhaT, tpiA*

Table 4. List of known and candidate cryptic genes, bold genes are known cryptic genes (Wren, 2003; Song et al., 2004), italic genes are candidate genes identified by CryptFind (Navid and Almaas, 2009).

meiotrophic mutations occurred accidentally or where selected for, however, it has been reported that in YP meiotrophic determinants are of secondary importance with regards to growth capacity of the organism (Dreyfus and Brubaker, 1978). Although absence of these processes do not hinder cellular growth under normal conditions, under unique circumstances, presence of these cryptic genes provides the cell with a reservoir of endogenous functionalities which can be activated through mutations and enhance a cell's adaptive capability (Hall et al., 1983). Interestingly, the meiotrophic losses play an important role in metabolism of YPS which needs to survive in austere conditions and scavenge any available nutrients that might be available. These can include uncommon sugars like rhamnose and melibiose. On the other hand YP can readily survive in its nutrient rich environment without the need to ferment these sugars. Accordingly, wild type strains of YP cannot utilize L-rhamnose as an energy source. However, a rare mutation (frequency of 2.6×10^{-11}) enables the bacteria to consume this methyl pentose under extraordinary conditions (Englesberg, 1957a). Recent theoretical analyses have identified the gene that encodes for the lactaldehyde dehydrogenase enzyme as the cryptic gene responsible for this meiotrophic phenomenon (Neary et al., 2007; Navid

[2] *glyA* was not reported as a possible cryptic gene in Navid & Almaas 2009 but was detected upon using CryptFind on a recently revised version of the model. The result is in agreement with some prior postulates (e.g. Brubaker 2006).

and Almaas, 2009). This result agrees with experimental observations that rhamnose positive strains of YP cannot fully oxidize rhamnose and that small amounts of lactic aldehyde are extracted from the supernatant fluid (Englesberg, 1957b).

Meiotrophic loses are not limited to carbon metabolism. Another such characteristic in YP is the absence of the urease enzyme due to the presence of a premature stop codon in the *ureD* gene (Sebbane et al., 2001). Urease plays a prominent role in nitrogen metabolism of YPS. Additionally, it has a critical role in oral transmission and subsequently pathogenicity of YPS. However, given its radically altered mode of infection, it is reasonable that YP lacks this activity. However, it worth noting that DNA microarray studies of YP have shown that some of the urease associated genes are expressed (Han et al., 2004; Motin et al., 2004). For example structural genes of *ureD* locus (operon *ureAB*) and the putative urea transporter (YPO2672) are upregulated following transition of the cell from 37°C to 26°C (Motin et al., 2004). Given that under some conditions the metabolic potential of YP can be enhanced by activation of cryptic genes, the identification and utilization of these added pathways have great potential for therapeutic treatment planning and drug target identification.

2. Errors in genome annotation

Genome annotation utilizes sequence homology to assign functions to genes in newly sequenced genomes. However, this process in not without its shortcomings. One of the most glaring of these deficiencies arises when few missense mutations inactivate the function of an enzyme. The problem arises from the fact that the investigator cannot differentiate between amino acid mutations in silent regions of the protein and those that

occur in key locations such as catalytic active sites. For example, the amino acids sequences of aspartase (AspA) enzyme in *E. coli* and YPS differ at about 50 positions and yet the kinetic parameters of these two enzymes are nearly the same (Viola et al., 2008). One the other hand, one or two missense mutations inactivate the AspA in YP although for every other location the amino acids sequence matches that of YPS AspA. Because of this strong sensitivity to specific mutations, analyses of annotated genomes of different strains of YP will show that they assign functionalities to products of cryptic genes and other proteins which experiments have shown to be inactive. Given that the primary foundation of FBA models are the network reconstructions derived from annotated genomes, development of fully predictive models necessitates identification and inactivation of these proteins.

3. CryptFind

In order to systematically find candidate cryptic genes that are responsible for YP's meiotrophic behavior, a computational tool called CryptFind was developed (Navid and Almaas, 2009). CryptFind utilizes FBA models' ability to accurately determine a gene's knockout phenotype to produce a list of candidate cryptic genes associated with a meiotrophic behavior. The simple two-step process of this approach involves:

1. Identifying all genes that are conditionally critical for growth in a minimal medium that only contains the nutrient source for which meiotrophic behavior has been reported.
2. Identify and remove from the above list those critical genes that are conditionally essential for the metabolism of any other nutrient source.

The remaining genes comprise the list of cryptic gene candidates. For example, if one is to determine the list of candidate cryptic genes responsible for YP's inability to ferment melibiose, CryptFind identifies an initial list of candidate cryptic genes ($\mathcal{C}$) by simulating system-wide gene knockouts for YP growing in a minimal medium containing only the obligate nitrogen, phosphorus and sulfur sources and melibiose as the sole carbon source. Next, melibiose is replaced by an array of different carbon sources and for each nutrient a new series of knockouts are simulated. Since the candidates in the resultant combined set of critical genes cannot be cryptic (or the metabolic capability to utilize that sugar will be lost), they are eliminated from $\mathcal{C}$. The remaining genes from $\mathcal{C}$ are the candidate cryptic genes for meiotrophic metabolism of melibiose.

To test the accuracy of CryptFind's predictions, it was applied to five meiotrophic pathways of YP for which the responsible cryptic genes where known. For every one of the meiotrophic pathways, CryptFind accurately identified the responsible gene as a candidate (Navid and Almaas, 2009). Additionally, CryptFind identified a set of genes that could be responsible for the three meiotrophic pathways for which the responsible cryptic genes have not been identified. Table 4 includes a list these possible cryptic genes.

4. Metabolic Robustness

Genome-scale FBA models of metabolism present a powerful tool for assessing a cell's strengths and weaknesses. This capability of constraint-based models was particularly useful for studying YP's robustness to genetic mutations. Since restrictions on the use of antibiotics makes large-scale experimental gene knockout screens of extremely

deadly organisms problematic. Model iAN818 was used to computationally determine the response of YP to drug interactions and single gene knockouts (SGKO) or synthetic lethal mutations (SLM). The results of these simulations are presented in Table 5.

The knockout simulations suggest that under all possible nutritional regimes, 74 of the 818 genes are critical for cellular growth. In a synthetic rich medium (TMH) (Straley and Bowmer, 1986), the number of critically important metabolic genes increase to 126, while in a nutritionally minimal media (MIN) the number rises to 168 (Navid and Almaas, 2009). Absence of oxygen makes the *dcuA* gene responsible for encoding the anaerobic C4-dicarboxylate transporter essential for cellular growth.

Since cells growing in nutritionally poor media need to produce nearly all of their metabolome *in vivo*, these cells (in comparison to those growing in TMH) are more susceptible to having a negative reaction in response to genetic mutations. Upon analyzing the additional SGKOs in the MIN medium, one discovers that over 80% of them are associated with metabolism of amino acids which are readily available in the TMH medium. Some of the other SGKOs are involved in production of cell envelope components and the anaerobic portion of pentose phosphate pathway (PPP). PPP in YP is particularly important due to the evolutionary loss of glucose 6-phosphate dehydrogenase enzyme in most strains of YP. Given PPP's primary role in producing important pentose precursors for a number of other pathways, any additional disruption can stop cellular growth.

Environment	Critical SGKO	Critical SLM
All conditions	74	39
Aerobic MIN	168	56
Aerobic TMH	126	61
Anaerobic MIN	170	74
Anaerobic TMH	127	77

Table 5. Computationally predicted cellular robustness of YP (strain 91001) to genetic mutations.

SLMs are pairings of genetic mutations that although they are individually harmless, in tandem they halt cellular growth. By scrutinizing the predicted SLMs for YP under a variety of different nutritional conditions, it has been discovered that over half (52%) of these lethal combinations are associated with alternate pathways which produce critical metabolites. The remainder of the SLMs include: pairs of critical isozymes (28%), transport and *in vivo* production processes (20%) for critical metabolites (Navid and Almaas, 2009).

V. Metabolism in the host environment

YP's utilization of metabolic pathways changes drastically upon its transfer from the flea vector to the mammalian host. The main difference between the two environments is temperature. While the flea gut temperature is 26°C, the mammalian host's normal temperature is 37°C. The environment in the host can be further divided based on concentrations of a number of cations, the most important of which is Ca^{2+}.

1. Low Calcium environments

Prior to discussing the details of various metabolic pathways, it is crucial to discuss an unusual nutritional requirement which is common to YP, YPS and YPE. This is because a number of metabolic effectors such as source of energy (Brownlow and Wessman,

1960), exogenous pH, as well as concentrations of CO_2 (Baugh et al., 1964) and some cations modulate this cellular behavior.

The cellular behavior dubbed “low-calcium response” (LCR) refers to the observation that following the transition from 26 to 37°C and in absence of Ca^{2+} (conditions resembling mammalian intracellular environments (Pollack et al., 1986), within one to two generations virulent strains of the bacteria undergo bacteriostasis. Although this mechanism is more rigorous in YP (Brubaker, 2007), it is important to the life cycle of both organisms. It has been suggested that LCR is necessary for adaptation of YP to the intracellular host environment (Brubaker, 1979). Mutations in a 70 kb plasmid (pCD) that is shared by all human pathogenic yersiniae eliminate the onset of LCR and simultaneously reduce the virulence of the bacterium (Portnoy and Falkow, 1981; Portnoy et al., 1983; Goguen et al., 1984). Other studies have shown that pCD carrying strains of YP inhibit cytokine production in the hosts and that this effect is mediated by some of the virulence factors whose expression is initiated by onset of LCR (Nakajima and Brubaker, 1993).

Start of LCR occurs under a narrow range of conditions. At 26°C, YP does require specific amounts of Ca^{2+} to grow. At 37°C, a minimal Ca^{2+} concentration of 2.5 mM represses the LCR. The requirement for Ca^{2+} can be replaced by equimolar concentrations of Sr^{2+} and Zn^{2+}; however addition of Mg^{2+} at concentrations similar to the inside of leukocytes (~20-40 mM) attenuates the LCR (Higuchi et al., 1959; Brubaker and Surgalla, 1964; Zahorchak et al., 1979). Presence of Na^{+} and dicarboxylic amino acids also increase the intensity of LCR (Fowler and Brubaker, 1994).

As can be expected, cessation of growth greatly alters the cellular metabolism. Studies have shown that one of the earliest metabolic processes downregulated during LCR is the production of RNA molecules. However, the same studies show that the mRNA portion of total cellular RNA does not change; and so concurrent to reduced production of RNAs, LCR leads to a decrease in rates of mRNA translation and degradation (Charnetzky and Brubaker, 1982). Additionally, LCR presenting cells display reduced adenylate energy charge which points to some negative regulation of bioenergetic pathways (Zahorchak et al., 1979).

Cellular deficiency in AspA might also be directly related to LCR. This loss results in a severe LCR that is characterized by buildup and excretion of L-aspartate (ASP). This deficit of metabolic carbon reduces the available stores of oxaloacetate which, as previously mentioned, leads to a reduced growth rate. Additionally, onset of LCR leads to expression of LcrQ a component of the type-three secretion system of the yersiniae. Recent studies have shown that this protein has an inhibitory effect on the enzyme phosphoenolpyruvate carboxylase (Ppc, EC 4.1.1.31) which in YP is one of the prominent pathways of oxaloacetate production (Schmid et al., 2009). Based on this result it can be surmised that the cell has augmented its metabolic capacity and instituted regulatory means to reduce its cellular growth via diminishing the intracellular oxaloacetate pool.

2. YP's Metabolism in Mammalian Blood

Some of the first studies of metabolism in YP showed that glucose uptake is greater when Ca^{2+} is present in the medium (Burrows and Bacon, 1956; Feodorova and

Golova, 2005). Subsequent analyses found that for environmental concentrations of Ca^{2+} similar to those in human blood, Ca^{2+} specific channels are activated and import this cation into the cell. This leads to a cascading series of reactions that result in initiation of DNA biosynthesis and bacterial growth(Charnetzky and Brubaker, 1982).

Gene-expression microarray analyses have been used to assess the role of temperature and Ca^{2+} on regulating different cellular processes (Motin et al., 2004). These results have shown that only genes on the pCD plasmid are regulated by both temperature and Ca^{2+}. Variations in Ca^{2+} concentration have negligible effects on the expression rate of chromosomal and pPCP and pMT plasmid genes.

However, in the presence of Ca^{2+}, a temperature shift to 37°C causes differential gene expression for 235 chromosomal genes. Approximately 80% of these changes in expression occur within 1 hour of the temperature change. The majority of these changes are transient and thus indicate that they have an immediate role in the bacteria's adaptation to temperature perturbation. A large number of the temperature regulated chromosomal genes are involved with cellular metabolism (Motin et al., 2004). Some of the genes associated with glycolysis and phosphorylation of gluconate are down-regulated. Meanwhile genes associated with transport of carbohydrates, pentose phosphate pathway and pentose metabolism are upregulated. The temperature change also induces oxidative metabolism by upregulating the expression of genes associated with the TCA cycle and cytochromes (Motin et al., 2004). Meanwhile the enzymes connected with the glyoxylate pathway are downregulated. Genes linked to nitrogen assimilation and amino acid biosynthesis (except phenylalanine) are downregulated. On the other hand, transition to 37°C leads to increased expression of genes related to

amino acid catabolism. Although a number of these upregulated catabolic genes are cryptic (e.g. *aspA* and *glyA*). Finally, genes associated with purine biosynthesis are downregulated while those linked to pyrimidine biosynthesis are upregulated (Motin et al., 2004).

Based on these results in can be argued that in environments resembling that of mammalian blood ($[Ca^{2+}]$>2.5mM and 37°C), YP initiates a metabolic regime of intense nutritional uptake and catabolism.

VI. Metabolic pathways

1. Glycolysis

Some of the earliest studies of metabolism in YP focused on pathways of carbohydrate consumption. These studies indicated that resting cells of YP utilize glucose primarily via Embden-Meyerhof-Parnus pathway and that alternate pathways such as pentose phosphate pathway (PPP), do not contribute to this process (Santer and Ajl, 1955a). Experimental observations and genomic analyses have shown that YP has the enzymatic capacity to catalyze all the constituent reactions of the glycolytic pathway (Parkhill et al., 2001; Brubaker, 2006). However, a number of observations seem to indicate that unlike YPS, in YP the traditionally recognized terminal step of this pathway, as catalyzed by the enzyme pyruvate kinase (EC 2.7.1.40), might not be as extensively used for conversion of phosphoenolpyruvate (PEP) into intermediary metabolites of the citric acid cycle (Brubaker, 2006). Instead YP tends to use the enzyme phosphoenolpyruvate carboxylase (Ppc) to carboxylate PEP into oxaloacetate (Baugh et al., 1964). A number of studies seem to indicate that balance of oxaloacetate in YP

is a critical factor in cellular growth. Any metabolic perturbation that results in accumulation of oxaloacetate tends to encourage cellular growth while depletion of oxaloacetate tends to have the opposite effect (Brubaker, 2006). Although utilization of pyruvate kinase does not have a negative effect on intracellular oxaloacetate pool, increased expression and use of Ppc allows for use of anaplerotic means to stimulate growth.

This deduction is strongly supported by the observation that presence of CO_2 or bicarbonate stimulates cellular growth (Baugh et al., 1964; Surgalla et al., 1964). In YP the reliance on Ppc could also alleviate some of the harmful consequences of aspartase deficiency. This is because absence of AspA means that any process that would convert oxaloacetate into aspartate would be diverting metabolic carbons into the production of a dead-end product (See page Figure 2 and the section on metabolism of dicarboxylic amino acids).

2. Pentose phosphate pathway

Initially, it was reported that all the constituent enzymes of PPP are present and active in YP, and that during the growth phase this pathway is used to provide the pentose phosphates that are necessary for production of biomass (Santer and Ajl, 1955b). These conclusions were based on experiments where using 1-^{14}C-glucose with avirulent Tjiwidej strain of bacteria, it was discovered that the amount ^{14}C labeled CO_2 released by growing YP was nearly 4 times greater than that liberated by resting cells. Presence of all enzymes of PPP was surmised since conversion of carbon 1 of glucose

to CO_2 results from oxidative decarboxylation by the pentose phosphate enzyme, phosphogluconate dehydrogenase (EC 1.1.1.44).

However, later studies with the virulent Alexander stain showed that although cell-free extracts of YP include a number of different enzymes of the pentose phosphate pathway, the activity of glucose-6-phosphate dehydrogenase (Zwf) (EC 1.1.1.49) could not be detected (Mortlock and Brubaker, 1962). This observation has been confirmed in over 50 different YP strains spanning diverse geographic locations and all virulent biovars (Brubaker, 2007). Contemporary sequence analyses of YP genome have found a mutation in the gene encoding this enzyme (a proline substitution in amino acid 161, (Chain et al., 2004)) which supports the latter observation.

Recently, it has been argued that the mutation of *zwf* does not fully eliminate the Zwf activity but instead results in YP producing only two isoforms of the enzyme which is one less than YPS (Feodorova and Golova, 2005). Consequently, routine enzymatic assay tend to show minute or undetectable amounts of this enzyme. They contend that although the activity of Zwf cannot usually be detected, the fact that it has been found intracellularly in dried bacteria (Santer and Ajl, 1955b) indicates that the cell still has the capability to produce this enzyme.

Other recent studies have detected the activity of Zwf in three pestoides strains of YP (avirulent to large mammals). Given the fact that most of the Zwf^+ strains of YP are avirulent (to large mammals), some have suggested that absence of this function might be related to bacterial virulence (Brubaker, 2006; Bearden et al., 2009; Bearden and Brubaker, 2010). It is argued that the role of Zwf as a biosynthetic enzyme is not critical

for cellular growth but is important toward balancing cellular redox status, which might have implications on bacterial virulence.

The cell has the ability to produce the necessary pentose it requires for growth, from hexose by reversing the normal reactions catalyzed by transketolase and transaldolase. It can also produce pentose from gluconate and thus bypass the first few steps of pentose phosphate pathway directly to produce 6-phosphogluconate through the action of gluconokinase (EC. 2.7.1.12). Furthermore, the cell can always import exogenous pentose from its nutritionally rich environment (Brubaker, 2006). Early analyses of metabolism of different energy sources showed that use of gluconate and ribose (but not glucose) favors production of virulence antigens (Brubaker and Surgalla, 1964). However, further analyses showed that if the pH of the medium is maintained at approximately 7, use of glucose, mannose and fructose actually stimulates greater antigen production (Brubaker and Surgalla, 1964). Despite the latter observation, there is in YP a “glucose effect” in that when grown with D-glucose in Ca^{2+} deficient media, the cells undergo significant lysis. Given Zwf’s role as a chief supplier to NADPH when cells are grown on hexose, its deficiency can have a serious effect on cellular LCR which is very important for bacterial virulence (Brubaker, 2007; Bearden et al., 2009; Bearden and Brubaker, 2010).

3. Anaerobic metabolism

Under normal circumstances, YP grows in an oxygen-rich environment. However, these bacteria are facultative anaerobes and can grow via fermentation. At 26°C expressions of a number of genes associated with anaerobic energy production (such

as fumarate and nitrate reductases) are upregulated (Han et al., 2004). Although this could indicate that in the flea gut environment oxygen is sparse and that YP's metabolism shifts to anaerobic respiration upon transmission into this medium, absence of concomitant increases in expression of regulatory proteins responsive to anaerobic conditions have led to postulation that the increase in expression of above mentioned metabolic enzymes is primarily due increased cellular growth at 26°C (in comparison to LCR conditions) (Han et al., 2004).

Whereas oxidative metabolism of glucose has been shown to produce very small quantities of organic acids, anaerobic carbon metabolism in YP is highly inefficient. In the absence of oxygen, metabolism of glucose will results in production of a number of organic acids, such as acetate, lactate and formate (Englesberg et al., 1954a; Englesberg et al., 1954b; Santer and Ajl, 1954). Overall, while oxidative metabolism of glucose results in nearly 60 percent assimilation of carbons into the cellular biomass, only 40 percent of the carbons are assimilated via anaerobic metabolism (Englesberg et al., 1954a). As can be expected, resting cells in anaerobic environments cease to maintain a viable store of enzymes associated with TCA cycle and associated processes for oxidative mode of energy production (Englesberg et al., 1954b).

4. Citric Acid Cycle & Oxidative metabolism

It has been reported that YP has a functioning TCA cycle (Englesberg and Levy, 1955). A number of observations indicate that given YP's nutrient rich environment, it frequently utilizes oxidative metabolism for its energy needs. For example, *in vitro* aeration of YP's medium increases its growth rate (Rockenmacher et al., 1952).

Growing YP rapidly oxidize glucose and pyruvate while oxidative metabolism of acetate, succinate, fumarate and malate proceed at a lower rate (Englesberg et al., 1954a). Finally, microarray gene expression data have shown that induction of cellular growth following the transition of cells from LCR to calcium rich and 26°C conditions are accompanied with increases in expression of genes associated with oxidative modes of energy metabolism (Han et al., 2004).

Even resting cells of YP, if grown aerobically, have a high rate of endogenous respiration (15-30% of respiration in presence of nutrients). This process proceeds independent of exogenous metabolism and thus the rate of O_2 uptake and CO_2 release is not a direct measure of metabolism of nutrients from the media (Santer and Ajl, 1954).

5. Glyoxylate Shunt

Although no enzymatic deficiencies have been reported, a number of experimental observations seem to indicate that the YP citric acid metabolism does not progress via the traditional reactions associated with the TCA cycle. Instead the bacteria seem to utilize the glyoxylate shunt as an alternate mean.

The glyoxylate shunt of TCA cycle is often used as an anabolic pathway for conversion of two-carbon compounds (such as acetate) into glucose via metabolism of acetyl-CoA. Experimental results support the notion that YP normally uses this pathway for its metabolic needs. First, it has been observed that YP can fully oxidize acetate with negligible production of α-ketoglutarate (Santer and Ajl, 1954). Additionally, YP extracts have a limited capacity to convert α-ketoglutarate into succinate (Brubaker, 2006).

Computational simulations of cellular metabolism also indicate that in rich media, there are a number of viable alternate pathways for metabolism of oxaloacetate (Navid and Almaas, 2009).

The critical enzymes for the glyoxylate bypass pathway are isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2). Unlike *E. coli* and other Yersiniae, YP maintains significant levels of both of these enzymes during growth on hexose and pentose molecules (Hillier and Charnetzky, 1981; Quan et al., 1982). In YP, two forms of isocitrate lyase have been detected. One form is active during growth on acetate and absent when alternate carbon sources are utilized. The other form of the enzyme is not constitutive but can be detected while growing on a variety of different carbon sources. Therefore, it has been postulated that the latter form might have a significant role that is not associated with the enzyme's traditional anaplerotic function (Hillier and Charnetzky, 1981).

Finally, microarray analyses of gene expression associated with the glyoxylate shunt show that three of these genes (*aceK*, *icl*, and *mas*) are upregulated upon in 37°C LCR conditions (Han et al., 2004). This could indicate that metabolism of two-carbon metabolites is more pronounced in the mammalian host; however, this hypothesis has not been verified.

Thus, it can be argued that, although YP posses the enzymatic means to proceed via traditional TCA cycle processes, it normally uses the glyoxylate shunt and bypasses some of the former's reactions. The reason for this deviation from the norm is not known. However, it has been hypothesized that given the glyoxylate shunt's role as the

mean to convert two-carbon organic compounds into TCA cycle intermediates, the metabolic change could serve as a mechanism to replenish oxaloacetate that is withdrawn from cellular metabolism via its conversion into ASP (Brubaker, 2006). This further supports the assertion that absence of AspA (which would have recycled ASP back into a TCA cycle intermediate) is a central factor in how the overall metabolism of the bacterium operates.

6. Amino acid metabolism

i. Missing amino acid biosynthesis pathways

The principal pathway for incorporation of NH_4^+ into cellular metabolome and biomass occurs through the action of the enzyme glutamate dehydrogenase (EC 1.4.1.4):

$$\alpha\text{-ketoglutarate} + NH_3 + NADPH + H^+ \leftrightarrow \text{L-glutamate} + H_2O + NADP^+$$

Following this initial step, the resultant glutamate (GLU) can be converted into other amino acids and intermediates of the TCA cycle through a number of different transamination reactions. Wild type (unselected) strains of YP unlike YPS cease to grow in media which have low levels of ammonium salts as the sole source of nitrogen (Rockenmacher et al., 1952). It has been proposed that this meiotrophic deficit can be attributed to mutationally induced inactivation of glutamine synthase (Brubaker and Sulen, 1971). The loss of this metabolic capability is yet another indication that YP's metabolism has begun to adapt to nutrient rich environments such as mammalian blood where only trace quantities of NH_4^+ can be found. Furthermore, it indicates that the bacterium prefers to import a large portion of its amino acid nutritional need from the surrounding medium.

In line with this metabolic program, YP has lost the capacity to produce a number of amino acids and under normal conditions requires their import for cellular growth. These amino acids are glycine (GLY) or threonine (THR), methionine (MET), phenylalanine (PHE), cysteine (CYS) (or sulfite or thiosulfate), isoleucine (ILE) and valine (VAL) (Brubaker, 2006). YPS does not have an obligatory requirement for import of these amino acids and thus it can be deduced that YP's transition to living in the nutritionally rich fluids has resulted in loss of capabilities which are essential for survival of its free living progenitor.

The requirement for import of either GLY or THR is meiotrophic and can be reversed by genetic mutation (frequency ~10^{-7}) (Brubaker and Sulen, 1971). Based on observations that serine (SER) cannot serve as a replacement for gly or thr and that YP's extracts in presence of ^{14}C labeled GLY do not produce radioactive SER, it has been proposed that the requirement for GLY/THR stems from a mutational loss of the enzyme serine hydroxylmethyl transferase (EC 2.1.2.1) (Brubaker and Sulen, 1971).

The mutation(s) that is responsible for the need to import of ILE and VAL is not known. However, it has been proposed that both requirements stem from deficiency in one common reaction (Englesberg and Ingraham, 1957). The *ilvN* gene (YPO2294 (CO92), y2717 (KIM)) which has been categorized as a pseudogene due to a mutational deletion (Chain et al., 2004) can serve as a candidate since it encodes one of the components of acetolactate synthase enzyme (EC 2.2.1.6). This enzyme is involved in biosynthesis of leucine (LEU), ILE and VAL. However, based on genomic information, there are a number of isozymes present in YP (see Figure 1). Because of this metabolic redundancy, computational modeling of YP's metabolism by using CryptFind, has not

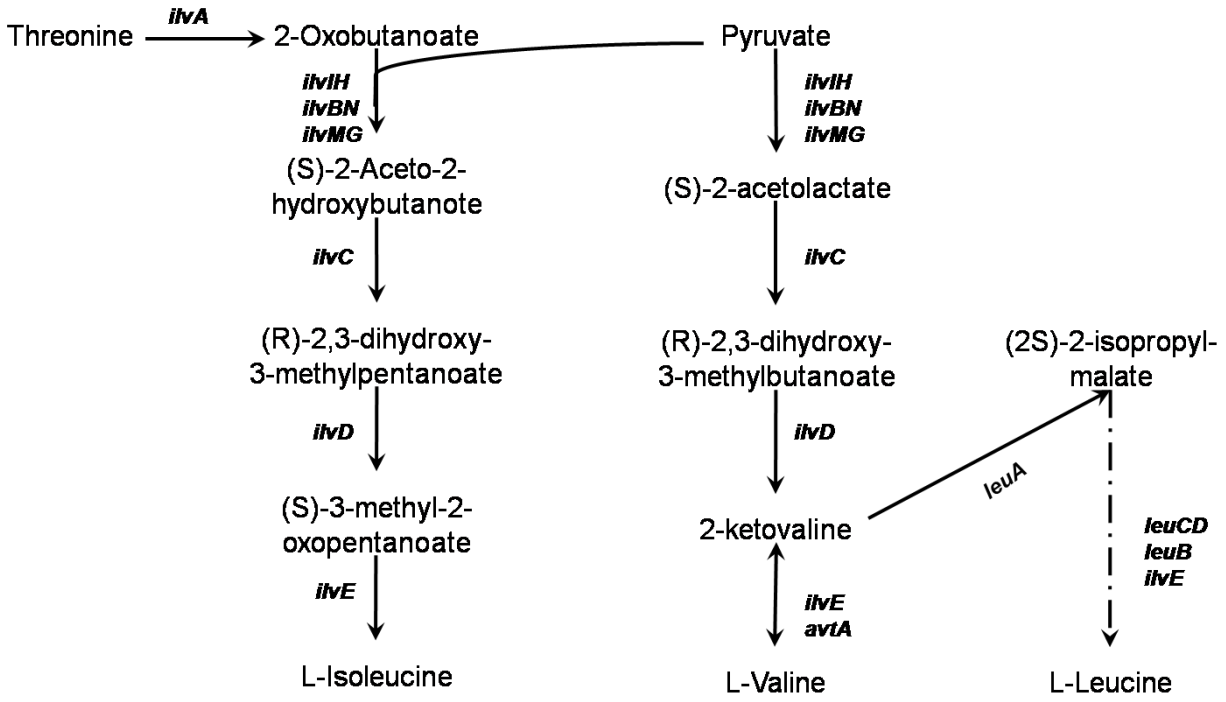

Figure 1. Metabolic pathway for biosynthesis of isoleucine, leucine and valine. The gene *ilvN* has been identified as a pseudogene (Chain et al., 2004). Theoretical predictions identify genes *ilvC* and *ilvD* as possible cryptic genes that are responsible for Y. pestis' meiotrophic loss of capability to produce isoleucine and valine (Navid and Almaas, 2009).

identified *ilvN* as a possible cryptic gene and instead has nominated two other genes (*ilvC* and *ilvD*) that are involved in biosynthesis of ILE, LEU, and VAL as the possible cryptic genes (see Table 4 and Figure 1) (Navid and Almaas, 2009). Given that import of LEU is not critical for cellular growth and does not remove the need for uptake of VAL or ILE, it can be reasoned that biosynthesis of LEU either occurs via a pathway that is distinct from that of the other two amino acids, or LEU can be produced irreversibly from ILE or VAL. We propose that in YP, imported VAL is degraded by valine-pyruvate aminotransferase (AvtA) (EC 2.6.1.66) to produce 2-ketovaline, a precursor of LEU (see Figure 1).

$$\text{L-valine} + \text{pyruvate} \leftrightarrow \text{2ketovaline} + \text{L-alanine}$$

ii. Use of amino acids as sources of carbon and nitrogen

Although only the import of the above mentioned amino acids is obligatory for cellular growth, a number of other amino acids can be metabolized by YP as carbon and nitrogen sources. For example, YP can rapidly catabolize SER via production of pyruvate and acetate intermediaries (Dreyfus and Brubaker, 1978).

Some studies have identified arginine (ARG) as one of the amino acid that cannot be catabolized by both YP and YPS (Dreyfus and Brubaker, 1978). However, metabolism of this amino acid is critical for YP's transitions between flea gut and host environments. Microarray gene expression studies show that at 37°C, some of the genes involved in ARG biosynthesis (*argABC*) and its interconversion to GLU (*astCADBE*) are upregulated (Han et al., 2004). The latter process releases CO_2 and ammonia as byproducts which can be interpreted as an added cellular need for ammonia as it transitions from flea gut to mammalian temperature. The need for ammonia might indicate increased amino acid interconversion. However, the consequences of raised intracellular ammonia concentrations are unknown (Han et al., 2004).

iii. Dicarboxylic amino acids

Dircarboxylic amino acids serve a number of important roles in bacterial physiology. Aside from their role as building blocks for proteins, these molecules also act as amine donor and acceptors, as well as intracellular signaling metabolites. Furthermore, due to their facile conversion into intermediates of the TCA cycle, GLU and ASP can serve as important supplementary nutrients that provide a cell with both the carbon and nitrogen that it needs for growth.

A comparison of the doubling times for YPS and YP (0.5 and 2 hours respectively) shows that despite metabolically adapting for growth in a rich media, YP growth significantly slower than its progenitor and therefore it must lack some important mechanism(s) that is associated with biomass production. It has been proposed that a lesion in the metabolism of dicarboxylic amino acids (Dreyfus and Brubaker, 1978) might account for YP's sluggish growth.

Experimental analyses of the uptake and consumption of various metabolites by YP and YPS demonstrate that the rates of catabolism for GLU, ASP and asparagine (ASN) are significantly lower in the former (Dreyfus and Brubaker, 1978). Genomic analyses have revealed that the *aspA* gene in different strains of YP contains at least one putative missense mutation (Parkhill et al., 2001; Deng et al., 2002; Chain et al., 2004; Zhou et al., 2004b; Chain et al., 2006). This gene encodes for the enzyme aspartase (AspA) (EC 4.3.1.1) which plays a prominent role in catabolism of ASP. It catalyzes the deamination of ASP into fumarate, an intermediate of the TCA cycle. Absence of this enzyme coupled with low turnover rate for some of the other enzymes involved in ASP and GLU metabolism (Dreyfus and Brubaker, 1978) severely limit the capacity of YP to convert these amino acids into bio-energetically useful metabolites.

Under some conditions in YP, ASP tends to be a dead-end metabolite. For example, the LCR of YP results in excretion of ASP as a byproduct of catabolism of exogenous glutamate (Brubaker, 2005, 2007; Viola et al., 2008). This unique metabolic feature results in extensive waste since each exported ASP represents the loss of 4 carbon nutrients which could have entered the cellular carbon metabolism via production of

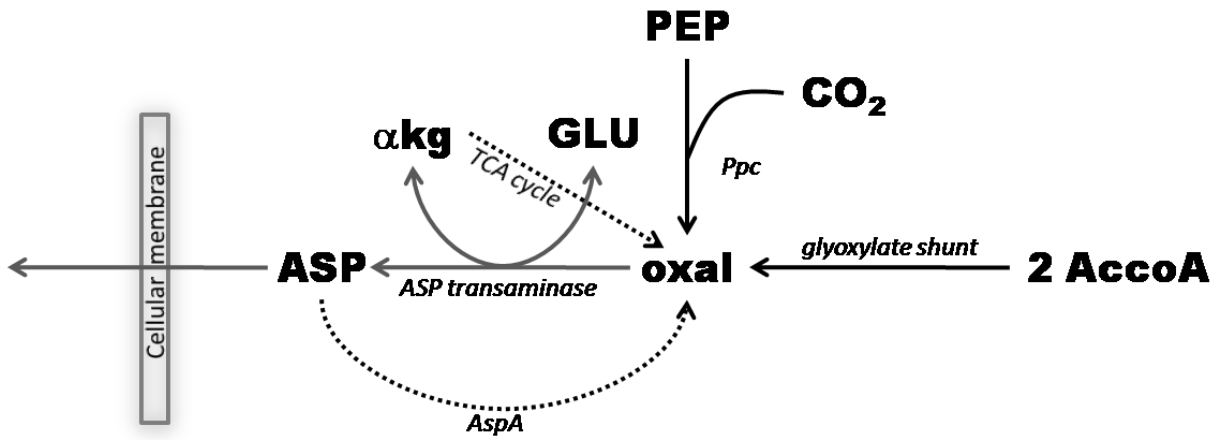

Figure 2. Prominent pathways of oxaloacetate production in *Y. pestis*. The black arrows denote those processes which favor oxaloacetate production. Grey arrows indicate those processes which reduce the intracellular pool of oxaloacetate. Dashed arrows represent processes in *Y. pestis* which are either missing or highly deficient.

fumarate and oxaloacetate (See Figure 2). Compensation for this nutrient loss explains the stimulatory effect of CO_2 on the growth of YP (Delwiche et al., 1959; Surgalla et al., 1964). CO_2 and bicarbonate can be converted into oxaloacetate through carboxylation of phosphoenolpyruvate by the enzymes phosphoenolpyruvate carboxykinase (PckA, EC 4.1.1.49) and phosphoenolpyruvate carboxylase (Ppc, EC 4.1.1.31) (Baugh et al., 1964).

An examination of Figure 2 shows that the only catabolic pathway for ASP in YP is its conversion to GLU via transfer of an amine to a molecule of α-ketoglutarate. However, the specific activity of the aspartate aminotransferase enzyme (EC 2.6.1.1) in YP is less than a tenth of that for YPS (Dreyfus and Brubaker, 1978). Furthermore, an increase in GLU concentration will shift the equilibrium in this reaction toward production of ASP at the cost of removing oxaloacetate out of the TCA cycle. As mentioned earlier, it has been postulated that any process which diminishes the intracellular pool of oxaloacetate has a deleterious effect on the cellular growth rate (Brubaker, 2006).

Accordingly, the absence of AspA in YP means that the primary pathway for conversion of dicarboxylic amino acids to TCA intermediates is the $NADP^+$ dependent deamination of GLU to α-ketoglutarate by glutamate dehydrogenase. Therefore, although the specific activity of glutamate dehydrogenase in YP is comparable to that of YPS (Viola et al., 2008), the excessive metabolic burden can overwhelm the capacity of this enzyme to metabolize dicarboxylic amino acids. Additionally, use of $NADP^+$ by glutamate dehydrogenase complicates the situation and further strains YP's metabolic mechanisms. Due to YP's deficiencies in the aerobic portion pentose phosphate pathway, the cell is forced to rely heavily on the activity of transhydrogenase enzyme to oxidize the resulting NADPH before it can be used for oxidative phosphorylation (Dreyfus and Brubaker, 1978).

It is worth noting that results of microarray gene-expression studies show that the *aspA* gene is normally transcribed in YP (Motin et al., 2004). However, it has been shown that this inactive AspA protein acts as a cross-reacting immunological material (CRIM) (Viola et al., 2008). CRIM is a protein produced from a mutated gene that does not have an enzymatic function but shows serological properties. Given that YP's AspA has the same substrate affinity for ASP as that of YPS AspA (Viola et al., 2008), it can be reasoned that the missense mutations that inactivate YP's AspA do not affect the primary structure of the enzyme that is responsible for substrate recognition. However, the k_{cat} for the YP's AspA is over 700 times smaller than that of its counterpart in YPS. Thus, it is evident that AspA's inactivity results from modifications in the catalytic residues of the active site (Viola et al., 2008).

Combining these results along with recent reports that YP uses the LcrQ protein to inhibit the activity of Ppc during LCR (Schmid et al., 2009), one can surmise that the bacterium by design reduces its oxaloacetate pool under some conditions. Although this is not indisputable evidence, the fact that the gene *aspA* is missing from the genomes of other deadly pathogens such as *Francisella tularensis* (Larsson et al., 2005), *Mycobacterium tuberculosis* (Fleischmann et al., 2002) and *Rickettsiae* (Andersson et al., 1998) strengthens the possibility for such a selection (Brubaker, 2007).

iv. Sulfur containing amino acids

YP requires MET and either CYS or thiosulfate for growth (Englesberg, 1952). Experimental evidence shows that this sulfur requirement of YP stems from at least two breaks in the pathway that converts sulfate to MET. The first of these fissures occurs at the conversion of sulfate and sulfite by sulfite oxidase (EC 1.8.3.1) and sulfite dehydrogenase (EC 1.8.2.1); and the second break occurs at the point of production of cystathionine from CYS by cystathionine gamma-lyase (EC 4.4.1.1). Genes that encode for these enzymes have not been identified in any of the sequenced YP genomes. However, experimental evidence has shown that a high rate mutation can overcome the latter pathway deficiency and allow for use of CYS, thiosulfate, sulfate or sulfite as sole sulfur sources. On the other hand, due to irreversibility of the cysthionine gamma-lyase reaction even this meiotrophic mutant cannot utilize MET as the sole sulfur source (Englesberg, 1952). In addition to the above candidates, additional examination of the annotated genomes of strains CO92 and KIM have shown that the *cysM* gene (YPO3011 and y1470 respectively) which encodes the cysteine synthase

enzyme is inactive in YP due to a frameshift (Chain et al., 2004). The exact consequences of this mutation are not known.

As with dicarboxylic amino acids, the metabolism of sulfur-containing amino acids is also perturbed when the cell adapts to new environments. The halt in cellular growth associated with LCR is marked with a reduction in expression of genes associated with organosulfur utilization and CYS biosynthesis (Han et al., 2004). It is postulated that this is due to the reduced energy needs of the bacteria when they are not growing. As previously mentioned, growth in YP is associated with a marked increase in the use of oxidative means for energy production. Bacterial c-type cytochromes are important constituent of electron transport mechanism and thus their biosynthesis is upregulated during periods of growth (Han et al., 2004). Maturation of cytochrome c requires use of cysteines. This can explain the observed increase in production of this amino acid during periods of growth (Han et al., 2004).

7. Nucleotide metabolism

One of the traditional established determinants of virulence in YP has been its ability for *de novo* biosynthesis of purines (Ferber and Brubaker, 1981). Examinations of the genome and *in silico* simulations indicate the cell has a full capacity to produce purine nucleotides from pentose precursor. This biosynthetic pathway is closely coupled to cellular growth and therefore the expression of genes associated with this pathway is downregulated in growth-limited LCR conditions (Han et al. 2004). Addition of ATP and GTP in the medium of LCR-presenting cells has been shown to promote cellular growth. These exogenous nucleotides are not transported and catabolized by the cell; therefore

it has been proposed that these external nucleotides promote cellular growth by sequestering free Mg^{2+} which amplifies YP's LCR (Zahorchak and Brubaker, 1982). However, this conclusion has been questioned since a mutation in the *lcrG* regulatory gene results in loss of LCR regulation by Ca^{2+} but does not affect regulation by nucleotides (Skryzpek and Straley, 1993). Based on this, it is possible that the regulatory role of nucleotides is separate from that of Ca^{2+} (Perry and Fetherston, 1997).

YP's purine metabolic pathways possess unique features in that they lack some of the capabilities found in other organisms for interconversion of nucleotides. For example, it has been experimentally shown that YP lacks the enzyme adenine deaminase (EC 3.5.4.2) which catalyzes conversion of adenine to hypoxanthine which subsequently is converted into guanine (Brubaker, 1970). Analyses of the annotated genomes support this experimental observation since the genes that encode for this enzyme are not present in any strains of YP or YPS. These metabolic limitations reduce YP's robustness to genetic mutations. For example, a mutation to the enzyme adenylate kinase, an enzyme which is involved in maintaining cellular adenine nucleotide homeostasis, drastically reduces bacterial growth and virulence (Munier-Lehmann et al., 2003).

As with purines, presence of pyrimidines in the Ca^{2+} deficient media at 37°C promotes growth of YP (Zahorchak and Brubaker, 1982). The specific mechanisms of this promotion are not known. However, it has been suggested that aside from promoting oxaloacetate production via carboxylation of PEP, another stimulatory effect of CO_2 and bicarbonate on YP's growth could be associated with the role of these compounds in

producing pyrimidines (Baugh et al., 1964). Bicarbonate can interact with glutamine in an ATP driven reaction catalyzed by carbamyl-phosphate synthetase (EC 6.3.5.5) to produce carbamyl phosphate, which is a precursor for production of pyrimidines (Jones et al., 1955).

Gene-expression microarray studies indicate that following a transition from 26°C to a Ca^{2+} rich environment at 37°C, the genes of YP associated with biosynthesis of pyrimidines are upregulated (Motin et al., 2004). This is an interesting metabolic phenomenon since this added production cannot be associated with growth; the expression of most other genes associated with production of nitrogenous compounds is repressed. This could suggest that pyrimidines under these conditions might have a unique regulatory role.

8. Fatty acid metabolism

Metabolism of fatty acids is critical for growth in some pathogenic bacteria (Moncla et al., 1983). Meanwhile presence of fatty acids has been shown to be toxic others (Okudaira et al., 1970; Hemsworth and Kochan, 1978). Given this dichotomy and due to the fact that human serum and interior of leukocytes contain high concentrations of fatty acids (Hemsworth and Kochan, 1978), it is critical for therapeutic purposes to ascertain the effect of metabolism of this class of metabolites on a pathogen.

Studies on metabolism of fatty acids in YP have provided conflicting results. Examinations in mice and guinea pigs showed that fatty acids are an anti-*Yersinia pestis* factor (Eisler and Heckly, 1968; Eisler and Von Metz, 1968). However, comparative analyses of metabolic pathways in a number of different gram-negative

bacteria (Sadovskaya et al., 2001), as well as some direct experimental measurements have shown that YP can constitutively import fatty acids and use them as sources of carbon (Moncla et al., 1983). It is hard to reconcile the proposition that fatty acids can poison YP with the fact that the natural niche for this organism is highly enriched in fatty acids. Additionally, there is a possibility that utilization of fatty acids during infection could be beneficial to the pathogen since it has been reported that rapid production of membrane components can improve a bacterium's survival within phagocytes (Melching and Vas, 1971). Consistent with these observations, studies indicate that some avirulent strains of YP lack the capability to import fatty acids (Ferber and Brubaker, 1981).

9. Iron transport mechanisms

Iron is essential for the survival of living organisms and a direct link has been established between its availability and YP's virulence (Burrows and Jackson, 1956). However, extraction of iron from host medium is much more involved than uptake of other metabolites. First, iron is not very soluble in biological fluids at neutral pH (Wooldridge and Williams, 1993). Furthermore, the majority of iron in mammals is incorporated in metalloproteins or iron storage proteins (Mietzner and Morse, 1994). In order to remove iron from the host medium, bacteria have devised a number of successful methods. The genome sequence of YP includes genes for eight putative iron and two heme transport systems (Deng et al., 2002).

One common mechanism of iron uptake by bacteria is secretion of siderophores, chelating compounds that have an extremely high affinity for Fe^{3+}. Given that the concentration of free iron in mammalian hosts is much less than that required for

bacterial growth and virulence, these compounds play a crucial role in pathogenesis of many bacteria. Based on this need, components of siderophore biosynthesis pathways, particularly salicylation enzymes, have been targeted for development of new drugs (e.g. (Ferreras et al., 2005; Miethke et al., 2006; Stirrett et al., 2008)).

In iron deficient media, YP secretes a virulence factor and tetracyclic siderophore known as yersiniabactin (Ybt). Ybt has a prominent role in iron uptake during early stages of infection (Bearden et al., 1997; Bearden and Perry, 1999; Fetherston et al., 1999). The genes involved in production of this chelating agent are located in the high pathogenicity island of YP (Carniel et al., 1996; Bearden et al., 1997; Perry et al., 1999) and encode the four proteins HMWP1, HMWP2, YbtE, and YbtU which comprise an assembly-line fusion of non-ribosomal peptide synthase and polyketide synthase components of the yersiniabactin synthetase complex. This grouping of proteins include seventeen functional domains of which twelve have catalytic roles while the other five act as carrier proteins (Miller et al., 2002). The ultimate task of this protein complex is to facilitate 22 chemical reactions which result in incorporation of five monomer units (one salicylate, three CYS and one malonyl molecules) into one Ybt.

In addition to the Ybt system, two other iron transport mechanisms in YP have been partially characterized. A hemoprotein uptake system allows for use of haemin or haem-protein complexes as an iron source. Proteins for this mechanism are encoded by the genes of haemin uptake locus (*hmu*). Based on uptake capability of mutants lacking this locus, the mechanism scavenges haemin as a source of both iron and porphyrins (Hornung et al., 1996). The Yfe system of YP belongs to an ABC family of transporters and specializes in uptake of both iron and manganese. This mechanism

plays a crucial role in iron acquisition in later stages of plague and is required for full virulence (Bearden and Perry, 1999).

10. Transmission factor metabolism

Extensive studies have been conducted into metabolism of biomolecules essential for mammalian infections (virulence factors). However, metabolism that is catalyzed by gene products critical for colonization of flea vector (transmission factors) is just as critical for perpetuation of YP's life cycle.

YP inhabit the midgut of fleas after they feed on the blood of septicemic mammals. In some fleas, the process of transmission of bacteria to mammals requires the blockage of the proventricular valve which separates the midgut from the esophagus (Hinnebusch, 1997). This often fatal blockage is mediated by the formation of a biofilm which is composed of a mass of bacteria surrounded by an extracellular matrix containing exopolysaccharides. The blockage occurs approximately two weeks after consumption of infected blood and ultimately prevents transfer of blood into flea's stomach. This obstruction compels the flea to continually attempt to feed. Unfortunately for the flea and surrounding mammals, these futile feedings will not sate the flea's hunger but result in infection of new hosts since each flea bite flushes bacteria into the a new bite wound. A grouping of genes designated as the hemin storage (*hms*) locus have been identified as crucial for formation of the YP biofilm and successful blockage of flea stomach (Hinnebusch et al., 1996; Darby et al., 2002; Jarrett et al., 2004). Microarray gene expression analyses have found that upon transition from 37°C to 26°C, the entire *hmsHFRS* operon in YP which encodes polysaccharide biosynthetic

proteins is upregulated (Han et al., 2004). Furthermore, *in vitro* analyses indicate that biofilm formation does not occur in mammalian hosts and is limited to low temperatures (21-28°C) akin to a flea's body temperatures (Perry et al., 1990; Pendrak and Perry, 1993; Jones et al., 1999).

One metabolic activity which has been shown to be essential for production of normal biofilms is the activity of phosphoheptose isomerase (GmhA). GmhA catalyzes the first step in the heptose biosynthesis pathway, the interconversion of sedoheptulose-7-phosphate and D-glycero-D-mannoheptose 7-phosphate (Brooke and Valvano, 1996). Although, heptose is not directly an element of YP biofilms, it is a conserved component of lipooligosaccharides and lipopolysaccharide. Thus, it is hypothesized that the functional failure of defective biofilms that are produced by *gmhA*$^-$ cells result from changes to the core components exopolysaccharides (Darby et al., 2005).

Microarray studies of YP gene expression during the process of biofilm formation have shown that genes associated with uptake of polyamines are also highly upregulated. However, deletions of these genes have no effect on the bacteria's ability to from biofilms and to block the flea's proventricular valve (Vadyvaloo et al., 2007). In absence of external putrescine, *in vivo* production of polyamines has been shown to be essential for formation of plague biofilm (Patel et al., 2006). Putrescine (1,4-diaminobutane) serves as the starting point for general polyamine biosynthetic production in most organisms. In bacteria the production of this important polyamine from amino acids can proceed through two different pathways. One pathway involves decarboxylation of an ornithine molecule into putrescine by ornithine decarboxylase (EC 4.1.1.17). The other pathway is composed of multiple steps starting with decarboxylation of arginine to

agamatine by arginine decarboxylase (EC 4.1.1.19), followed by conversion of agamitine to N-carbamoylputrescine by agamatine deiminase (EC 3.5.3.12) which then is disintegrated into putrescine via N-carbamolyputrescine amidohydrolase (EC 3.5.1.53). A third pathway involving direct conversion of agamatine into putrescine that is catalyzed by agmatinase (EC 3.5.3.11) has been detected in *E. coli* (Szumanski and Boyle, 1990), however, genomic analyses suggest that this enzyme is not present in YP and only the multistep conversion of agamatine is used for polyamine biosynthesis (Patel et al., 2006).

Polyamines putrescine and spermidine also play important roles in a number of other cellular processes. They have a part in modulating transcription and translation of DNA and mRNA (Tabor and Tabor, 1985; Igarashi and Kashiwagi, 2000; Yoshida et al., 2004). Additionally, in *E. coli* polyamines have been shown to activate the *oxyR* and *katG* stress response genes (Tkachenko et al., 2001). Finally, there is some evidence that putrescine might have a role in regulating bacterial differentiation and intracellular signals that synchronize multi-cellular actions (Sturgill and Rather, 2004).

Finally, it should be noted that some recent studies have shown that infected fleas can transmit YP to a host as early as one day after intake of infected blood and hence blockage of flea stomach might not be as critical as some have suggested (Eisen et al., 2006; Eisen et al., 2007; Eisen et al., 2009).

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